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Iskender, Ilker ; Arni, Stephan ; Maeyashiki, Tatsuo ; Citak, Necati ; Sauer, Mareike ; Monné Rodríguez, Josep M ; Frauenfelder, Thomas ; Opitz, Isabelle ; Weder, Walter ; Inci, Ilhan

Abstract: Objective: Improvement in ex vivo lung perfusion (EVLP) protocols could increase the number of donors available for transplantation and protect the lungs from primary graft dysfunction. We hypothesize that perfusate adsorption during EVLP reconditions the allograft to ischemia-reperfusion injury after lung transplantation. Methods: Donor pig lungs were preserved for 24h at 4°C, followed by 6h of EVLP according to the Toronto protocol. The perfusate was additionally adsorbed through a CytoSorb adsorber in the treatment group, whereas control lungs were perfused according to the standard protocol (n = 5, each). EVLP physiology and biochemistry were monitored. Upon completion of EVLP, a left single lung transplantation was performed. Oxygenation function and lung mechanics were assessed during a 4-hour reperfusion period. The inflammatory response was determined during EVLP and reperfusion. Results: The cytokine concentrations in the perfusate were markedly lower with the adsorber, resulting in improved EVLP physiology and biochemistry during the 6-hour perfusion period. Post-transplant dynamic lung compliance was markedly better during the 4-hour reperfusion period in the treatment group. Isolated allograft oxygenation function and dynamic compliance were continued to be superior in the adsorber group at the end of reperfusion accompanied by a markedly decreased local inflammatory response. Conclusions: Implementation of an additional cytokine adsorber has refined the standard EVLP protocol. Furthermore, cytokine removal during EVLP improved immediate post-transplant graft function together with a less intense inflammatory response to reperfusion in pigs. Further studies are warranted to understand the beneficial effects of perfusate adsorption during EVLP in the clinical setting.

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Perfusate-adsorption during *ex vivo* lung perfusion improves early post-transplant lung function

Ilker Iskender, MD, MSc,[a] Stephan Arni, PhD,[a] Tatsuo Maeyashiki, MD,[a] Necati Citak, MD,[a] Mareike Sauer, DVM,[b] Josep Monné Rodriguez, DVM,[c] Thomas Frauenfelder, MD,[d] Isabelle Opitz, MD,[a] Walter Weder, MD,[a] Ilhan Inci, MD,[a]

From the [a]Department of Thoracic Surgery, University Hospital Zurich–University of Zurich, Zurich, Switzerland; [b]Department of Surgical Research, University Hospital Zurich–University of Zurich, Zurich, Switzerland; [c]Department of Veterinary Pathology, University of Zurich, Zurich, Switzerland; and [d]Institute of Diagnostic and Interventional Radiology, University Hospital Zurich–University of Zurich, Zurich, Switzerland.

Corresponding author: Ilhan Inci, MD

Address: Department of Thoracic Surgery, University Hospital Zurich, Raemistrasse 100, Zurich 8091, Switzerland.

Email: ilhan.inci@usz.ch

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Glossary of Abbreviations

BWF	=	bronchial wash fluid
EVLP	=	*ex vivo* lung perfusion
IL	=	interleukin
IL-1ra	=	interleukin-1 receptor antagonist
IR	=	ischemia-reperfusion
PGD	=	primary graft dysfunction
PaO_2/FiO_2	=	partial pressure of arterial oxygen/fraction of inspired oxygen
SD	=	standard deviation

Central Message

Adsorption of inflammatory mediators during *ex vivo* lung perfusion improves immediate post-transplant graft function by decreasing inflammatory response to reperfusion in pigs.

Perspective statement

Current *ex vivo* lung perfusion protocols are limited by short perfusion times and scarce treatment options. Advancements in the perfusion protocols may improve the quality of donor lungs and result in increased rates of utilization. Implementation of an additional broad spectrum adsorber in perfusion circuits not only refines the current protocols, but also improves short-term graft function.

Abstract

Objective: Improvement in *ex vivo* lung perfusion (EVLP) protocols could increase the number of donors available for transplantation and protect the lungs from primary graft dysfunction. We hypothesize that perfusate adsorption during EVLP reconditions the allograft to ischemia-reperfusion injury after lung transplantation.

Methods: Donor pig lungs were preserved for 24h at 4°C, followed by 6h of EVLP according to the Toronto protocol. The perfusate was additionally adsorbed through a CytoSorb adsorber in the treatment group, whereas control lungs were perfused according to the standard protocol (n = 5, each). EVLP physiology and biochemistry were monitored. Upon completion of EVLP, a left single lung transplantation was performed. Oxygenation function and lung mechanics were assessed during a 4-hour reperfusion period. The inflammatory response was determined during EVLP and reperfusion.

Results: The cytokine concentrations in the perfusate were markedly lower with the adsorber, resulting in improved EVLP physiology and biochemistry during the 6-hour perfusion period. Post-transplant dynamic lung compliance was markedly better during the 4-hour reperfusion period in the treatment group. Isolated allograft oxygenation function and dynamic compliance were continued to be superior in the adsorber group at the end of reperfusion accompanied by a markedly decreased local inflammatory response.

Conclusions: Implementation of an additional cytokine adsorber has refined the standard EVLP protocol. Furthermore, cytokine removal during EVLP improved immediate post-transplant graft function together with a less intense inflammatory response to reperfusion in pigs. Further studies are warranted to understand the beneficial effects of perfusate

adsorption during EVLP in the clinical setting.

The implementation of *ex vivo* lung perfusion (EVLP) in donor lung management is the most significant advancement in lung transplantation over the last decade.[1] Initially EVLP was introduced as a second-look procedure to high-risk donor lungs prior to transplantation. Growing experience with EVLP has assured its safety and efficacy in lung transplantation.[2] Centers across the world have successfully adopted the procedure and reported increased utilization of donor lungs.[3,4]

Other than donor lung assessment, EVLP also has a role in donor lung preservation. Accumulating evidence suggests that extension of donor ischemic time with an additional EVLP between the two cold static preservations does not have a negative impact on early outcomes of lung transplantation.[5,6] Moreover, EVLP driven preservation of the standard criteria donor lungs have been shown to be effective and safe regardless of the perfusion protocols.[7,8]

One of the limitations of the current EVLP protocols is the short perfusion times. To our knowledge, EVLP for more than 12h has not been reported so far using the three widely used EVLP protocols in the clinical setting. Several studies have been performed to optimize the EVLP protocols looking at the roles of atrial pressure,[9] synchronous bronchial artery circulation,[10] cellular and acellular perfusates,[11] continued perfusate exchange,[12] negative pressure ventilation,[13] perfusate oxygen concentration,[14] continuous perfusate adsorption,[15] and positioning of donor lungs.[16] Combination of these strategies may result in prolonged EVLP times.

EVLP simulates ischemia and reperfusion (IR) processes in lung transplantation, which is characterised by the release of inflammatory mediators into the perfusate.[17] Kakishita et al have shown the feasibility of cytokine removal with an adsorbent membrane during

EVLP.[18] Recently, we also demonstrated beneficial effects of perfusate adsorption during EVLP using a novel membrane (Cytosorb®) in a prolonged cold ischemic injury model in pigs.[15] In addition to marked cytokine removal, continuous perfusate adsorption improved the EVLP physiology and biochemistry in this setting.[15] Moreover, increased expression of pro-inflammatory cytokines during EVLP has been shown to correlate with higher primary graft dysfunction (PGD) rates after lung transplantation.[19] The present study is based on the hypothesis that perfusate adsorption during EVLP reconditions the allograft to IR induced injury after lung transplantation and improves short-term graft function. Improvement in EVLP could increase the number of donors available for transplantation and protect the lungs from PGD.

METHODS

All animals received humane care during experiments in accordance with the updated "The Guide for the Care and Use of Laboratory Animals" (8th Edition, US National Research Council). The animal use protocol was approved by the Veterinary Authorities of the Swiss Kanton of Zurich. Female domestic pigs were used.

Pharmacokinetics of meropenem and methylprednisolone in EVLP perfusate from a previous trial

The CytoSorb® adsorber contains microporous beads, which are capable of adsorbing broad spectrum of mediators, including drugs.[20] Thus, we first decided to analyse the perfusate samples collected at baseline, 1h, 3h, and in 3h intervals thereafter during our previous 12h EVLP trial.[15] The two target drugs, Methylprednisolone (Medrol®) and Meropenem (Meronem®), were extracted from perfusate samples using a methanol

precipitation. For pharmacokinetics, samples were analyzed by liquid chromatography-mass spectrometry using selected-reaction-monitoring assays. The system consists of a Thermo TSQ-Quantiva coupled to a Waters M-class UPLC. The LC was set up in reverse phase mode (C-18, 150µm x 5cm). External calibration curves, with a concentration range of 10-500 nM, were included. Quantification was performed using the Xcalibur Quan Browser software (Thermo Fisher Scientific, Waltham, MA).

Lung retrieval, EVLP, and lung transplantation procedures

The experimental procedures were previously described in detail elsewhere.[21] The lungs were kept in a cold room for 24h at 4°C following pneumoplegia with Perfadex (XVIVO Perfusion, Göteborg, Sweden). EVLP was performed according to the Toronto protocol for 6h.[2] Perfusions were performed with or without the CytoSorb® adsorber, commercially available for use in Europe with a CE mark, (CytoSorbents Inc, Monmouth Junction, NJ) according to an established methodology in a randomized fashion (n = 5, each).[15] Recipient operation was started at 5h of EVLP. After a thoracotomy, the right pulmonary artery was encircled followed by a left pneumonectomy. Orthotopic left single lung transplantation was then performed and the recipients were observed for 4h.

Physiological assessment during EVLP and after transplantation

The allograft function was assessed hourly during EVLP and after reperfusion by means of pulmonary gas exchange, lung mechanics and vascular pressures. At the end of 4h of reperfusion, the allograft was challenged with a contralateral pulmonary artery occlusion for 5min, followed by clamping of the right main bronchus to a target pressure of 30 cmH_2O for evaluation of the isolated allograft mechanics.

Perfusate, plasma, bronchial wash fluid (BWF), and tissue collection and analyses

Perfusate and plasma samples were collected hourly. The Epoc blood analysis system (Epocal, Inc., Ottawa, ON, Canada) was used to measure perfusate and blood gases and for biochemistry.

At the end of EVLP and transplantation, a bronchoalveolar wash was performed with 20 mL of cold saline from the lower lobe segments of the right and left lungs, respectively. Approximately 15 mL were recovered during each wash. The recovered wash fluid was subjected to cytological assessment at the hospital's core laboratories, using May–Grünwald–Giemsa stained cytological specimens and subsequently processed as previously described for further analysis.[22] Similarly, lung tissue samples were also collected at the end of each procedures.

Assessment of the inflammatory response

The inflammatory response was analysed in perfusate, plasma, and BWF samples using Milliplex MAP Porcine Cytokine/Chemokine Magnetic Bead Panel (EMD Millipore, Billerica, MA) by a luminex laboratory at the Mouse Metabolic Evaluation Facility of the University of Lausanne (Switzerland). Additionally, tissue expressions of interleukin (IL)-1β and IL-8 were analysed as previously described.[23]

Protein assay

The Pierce micro BCA kit was used to measure the total protein concentrations in perfusate, BWF, tissue, and plasma samples according to the manufacturer's instructions (Thermo Scientific, Rockford, IL).

Radiologic assessment of the lungs

Lung X-rays taken at the end of EVLP were evaluated by a radiologist in a blinded fashion as previously described.[15]

Histological assessment of the lungs

Tissue samples taken at the end of EVLP and transplantation were stained with hematoxylin and eosin and blindly assessed by two veterinary pathologists for any pathological changes. The most prominent features observed in the transplanted lungs were used to develop a scoring system. This was as follows: A) Evidence of neutrophil recruitment into the lungs, represented by the presence of neutrophils within vessels (1: moderate numbers, 2: high numbers, 3: vessels packed with neutrophils); B) Evidence of neutrophil emigration into the tissue (1: neutrophil rolling along vascular endothelial cells, 2: neutrophils within vessel walls, 3: neutrophils immediately outside vessels); and C) Evidence of neutrophils within the tissue, represented by the presence of individual neutrophils within alveolar lumina (1: in rare alveoli, 2: in occasional alveoli, 3: in numerous alveoli).

Statistical analyses

All results are expressed as mean ± standard deviation (SD). Mann–Whitney U-test was utilized, where data were non-continuous. Two-way analysis of variance for repeated measures was utilized, where such data contained a time component. Statistical analyses were performed with PRISM 5 software (GraphPad Software, Inc., La Jolla, CA). Differences were considered significant when the *p*-value is less than 0.05.

RESULTS

The effects of the adsorber on pharmacokinetics of meropenem and methylprednisolone under EVLP conditions

Meropenem levels were diminished by more than 50% of the baseline at 1h and remained

stable throughout perfusions in the control group (Figure 1A). Interestingly, methylprednisolone levels were sustained at around 80% of the baseline during the 12h of EVLP period in the control group (Figure 1B). These results may indicate that tissue absorption of meropenem was much higher as compared to prednisolone during EVLP. However, the perfusate concentrations of meropenem and methylprednisolone were markedly decreased in the adsorber group compared with the control group ($p<0.001$, Figure 1A and 1B). The drug removal effect of the adsorber was more prominent for methylprednisolone than meropenem.

Reproducibility of EVLP part

Pre-retrieval characteristics of donor animals

Donor body weight (control: 29.8±3.6 vs adsorber: 28.4±1.5 kg; p=0.67), donor static compliance (control: 23.6±2.5 vs adsorber: 25.3±3.5 mL/mbar; p=0.42) and cold ischemic time (control: 24.1±0.3 vs adsorber: 24.3±0.3 hours; p=0.42) did not differ significantly between the two groups. Of note, the last partial pressure of arterial oxygen/fraction of inspired oxygen (PaO_2/FiO_2) ratio was markedly higher in the control group compared with adsorber (65.9±3.5 vs 57±4.8 kPa; p=0.008).

EVLP physiology and biochemistry

During the 6h EVLP period, peak airway pressure was significantly lower in the adsorber group (p=0.045, Figure 2A). Dynamic compliance (p=0.27, Figure 2B) and pulmonary vascular resistance (p=0.26, Figure 2C) did not differ significantly between the two groups. Moreover, the pulmonary veno-arterial oxygen pressure gradient was markedly better in the adsorber group (p=0.016, Figure 2D).

Electrolytes, such as hydrogen, potassium, and calcium continued to accumulate in the

perfusate during the 6-h EVLP period (Figure 2E-G). However, a trend towards a more physiologic pH (p=0.055, Figure 2E), and markedly decreased potassium (p=0.003, Figure 2F) and calcium (p<0.001, Figure 2G) levels was observed in the adsorber group.

Similarly, a gradual decrease in perfusate glucose concentrations combined with high lactate levels were the characteristics of pulmonary metabolism during EVLP (Figure 2H-I). There was a trend towards lower glucose consumption rates (p=0.059, Figure 2H) and a significant decrease in lactate production in the adsorber group compared to the controls (p=0.004, Figure 2I).

Inflammatory response

Perfusate samples collected at 3h and 6h of EVLP were analyzed using a multiplex kit. Overall, the perfusate levels of IL-1α, IL-1β, IL-1 receptor antagonist (ra), IL-6, IL-8, IL-10, IL-12, and IL-18 were found to gradually increased over time in the control group (Table 1). However, all analytes were significantly lower in the adsorber group during the 6-hour EVLP period. Furthermore, the perfusate total protein concentration was also markedly lower in the adsorber group (80.2±6 vs 64.5±8.3 mg/mL; p=0.032).

End-EVLP assessments

The cytological examination found that macrophages were the predominant cell type in the BWF in both groups (Control: 85.9±9.3 vs Adsorber: 89.1±5.7 percent; p=0.68). The following parameters did not differ significantly, but they were lower the treatment group: Perfusate consumption (Control: 536±163 vs Adsorber: 394±119 mL; p=0.31) and radiologic lung injury scores (Control: 6.4±2 vs Adsorber: 4.4±1.5; p=0.205). In both groups, the histological changes were comparable and restricted to mild to moderate subpleural and interstitial edema and the presence of apoptotic leukocytes within vessel

lumina and, to a variable extent, within the occasionally observed mild bronchus associated lymphatic tissue (Supplement Figure A).

Outcomes of lung transplantation

Baseline characteristics of recipient animals

Recipient body weight (control: 32.2±2.2 vs adsorber: 32±1.7 kg; p=1), recipient static compliance (control: 25.8±3.8 vs adsorber: 25±2.6 mL/mbar; p=0.92), the PaO_2/FiO_2 ratio before thoracotomy (control: 64.6±6 vs adsorber: 61.5±6 kPa; p=0.69), and warm ischemic time (control: 71.4±8.7 vs adsorber: 76±4.2 minutes; p=0.45) did not differ significantly between the two groups.

Post-transplant physiological parameters

Recipient animals were supported hemodynamically using ringer's lactate solution throughout the procedure. Overall, mean arterial pressures were comparable between the two groups during the experimental procedures (p=0.135, Figure 3A).

Dynamic pulmonary compliance was significantly higher throughout reperfusion in the adsorber group (p=0.031, Figure 3B). Although not reaching a statistical significance, systemic arterial (p=0.099, Figure 3C) and pulmonary venous (p=0.175, Figure 3D) oxygenation function were superior in the treatment group.

After clamping the right pulmonary artery, allograft continued to provide a superior gas exchange function in the treatment group compared with control (37±17 vs 61.8±11.8 kPa; p=0.056, Figure 3E). Similarly, isolated dynamic compliance was significantly better in the adsorber group (7.7±3.2 vs 12.9±2.2 mL/mbar; p=0.032, Figure 3D).

Post-transplant inflammatory response

The inflammatory response was assessed in plasma collected at baseline, 2h, and 4h of

322 reperfusion and BWF samples were collected at autopsy for 8 analytes. The plasma levels
323 of IL-1ra, an acute phase protein, were significantly lower in the absorber group during the
324 4-h reperfusion period (p=0.009, Figure 4). Other analytes in the plasma did not differ
325 significantly between the two groups and the results are not presented. When we looked at
326 the BWF samples, except for IL-1α, measurements of all other analytes were lower in the
327 treated lungs (Table 2). For IL-1ra, IL-6, and IL-8, the difference was statistically
328 significantly. In parallel with the BWF, tissue expressions of IL-1β (control: 4.4±0.8 vs
329 adsorber: 3.5±0.6 ng/mL; p=0.056) and IL-8 (control: 213±125 vs adsorber: 137±76
330 pg/mL; p=0.31) were lower in the treatment group (Figure 5, upper panel). On the other
331 hand, total protein concentration of plasma (control: 54.9±6.6 vs adsorber: 52.9±3.4
332 mg/mL; p=0.84), BWF samples (control: 62.9±52.4 vs adsorber: 33.8±42.4 mg/mL;
333 p=0.22), and tissue (control: 3.2±0.1 vs adsorber: 3±0.3 mg/mL; p=0.31) were comparable
334 between the two groups.
335 *Post-transplant assessments*
336 BWF cytology at autopsy showed predominantly neutrophilic response in both groups
337 (Control: 59.3±23.9 vs Adsorber: 44.2±22.4 percent; p=0.42). The histological assessment
338 of the lungs collected during the autopsy showed consistent changes in response to
339 reperfusion, reflecting the recruitment of neutrophils into the lung. All animals exhibited
340 moderate or high numbers of neutrophils in vascular lumina, in one animal in the control
341 group several vessels were packed with neutrophils (Animal #5, Figure 5C). Vascular
342 endothelial cells were generally found to be activated (Supplement Figure B); this
343 coincided with evidence of neutrophil rolling along the endothelial cells, and in several
344 animals also with proof of neutrophil emigration (Supplement Figure C), represented by

the presence of neutrophils in arterial walls and even in the perivascular tissue. Lastly, in most lungs' neutrophils were found in alveoli (Supplement Figure D). The overall microscopic lung injury scoring was comparable between the two groups (5.2±2.7 vs 4.2±1.6; p=1, Figure 5C). However, the presence of individual neutrophils within alveolar lumina (1.9±1 vs 1±1; p=0.29, Figure 5D) were lower, indicating a less intense inflammatory response in the adsorber group. Furthermore, the discordance between the two-pathologists related to the microscopical lung injury are given in detail in supplementary Table.

DISCUSSION

In the present study, we have further tested the beneficial effects of perfusate adsorption during EVLP on short-term post-transplant lung function in a pig EVLP lung transplant model. With this strategy, we have repeatedly shown that EVLP physiology and biochemistry were significantly improved.[15] Furthermore, cytokine filtration during EVLP resulted in the preservation of post-transplant graft function, represented by enhanced gas exchange and lung mechanics, and a less intense inflammatory response to IR-injury in pigs (Figure 6). We conclude that implementation of an additional cytokine adsorber has refined the standard EVLP protocol and maybe beneficial to protect donor lungs from unwanted effects of PGD in lung transplantation.

Other than cytokines, the adsorber removes a wide range of molecules such as damage- and pathogen-associated molecular patterns, metabolites, hormones, proteins, and drugs.[20] We have previously shown a certain albumin removal effect of the adsorber under EVLP conditions.[15] Antibiotics, steroids, and heparin are generally added to the prime solution in

EVLP protocols. Thus, we first determined the pharmacokinetics of meropenem and methylprednisolone in perfusate samples collected during our previous trial. The impact of the adsorber on drug removal from perfusate was remarkable for both meropenem and methylprednisolone, but the latter more prominently in this study. Interestingly, tissue adsorption of meropenem as compared to methylprednisolone was much higher in the control lungs. To our knowledge, the impacts of the empiric use of antibiotics and steroids in EVLP has not been studied elsewhere, thus the clinical relevance of the reduced levels of these substances remains unclear. Therefore, we have decided not to change the current regime in the present study. However, dose adjustments are advised to reach therapeutic drug levels during treatment with CytoSorb® in EVLP setting.

Reproducibility in animal research and translation from bench to bedside are continuing to challenge the scientific community. Several guidelines have been published to improve reproducibility in research, yet the practice remains largely unchanged.[24] We have previously shown the beneficial effects of continued perfusate adsorption with CytoSorb® during a 12h EVLP period in pigs in a proof-of-concept study.[15] In the present study, we have decided to perfuse the lungs for 6h only to mimic the clinical scenario. Furthermore, at this time point, significant cytokine clearance was achieved, accompanied by improved EVLP physiology during the initial trial.[15] Overall, the previous outcomes of the EVLP part are reproduced in the present study. The treated lungs exhibited less intense pulmonary edema, determined by decreased airway pressures, during the EVLP period; lesser pulmonary compliance and radiologic lung injury scores provide further evidence of decreased lung edema. The short perfusion time may have prevented these outcomes reaching a statistical significance and subsequent histological evidence of quantitative

differences. Remarkably, oxygenation function of the treated lungs during EVLP was markedly better in the present study. Yet, gas exchange function during EVLP has been suggested as the least important parameter for assessing graft quality in acellular perfusion method.[25] Other than physiological parameters, perfusate analyses provide insights regarding the quality of donor lungs. Glucose consumption and lactate production have been suggested as predictive markers for decision making during EVLP.[26] In parallel with our previous findings, perfusate adsorption lead to markedly improved the pulmonary metabolism in this study. In combination with physiological and biochemical parameters, novel biomarkers are needed to accurately predict post-transplant outcomes of EVLP assessed donor lungs.

Accumulating evidence suggests that EVLP simulates IR injury in lung transplantation. Restoration of pneumocyte function under normothermic conditions induces enhanced cytokine expression during EVLP,[17] which is similar to the inflammatory response that is seen after reperfusion with the recipients' blood in lung transplantation.[27] Furthermore, an enhanced inflammatory response during EVLP has been shown to correlate with poor outcomes related to PGD.[19] In line with our previous findings, the adsorber safely and effectively cleared the cytokines from the perfusate, resulting in improved EVLP physiology in this study. Kakishita et al also showed the feasibility of cytokine removal during EVLP, yet the EVLP physiology did not improve under the experimental conditions they used.[18] The adsorber device used in their study is comprised of a cellulosic bead modified with a hexadecyl ligand. It is indicated for selective elimination of β2-microglobulin from the circulating blood of patients with dialysis-related amyloidosis.[28] This differs from CytoSorb®, where there are no ligands attached to the polymer, but which

functions on size selectivity and surface adsorption. Furthermore, the experimental conditions of the two studies were very different. The EVLP was initiated after a short warm-ischemic time, whereas the present study used a clinically relevant prolonged cold ischemic injury model.[29] However, donor lungs undergo different types of insults, such as brain death, aspiration, pneumonia, and barotrauma in the clinical setting.[30] The broad spectrum adsorber used in this study has a capability to adsorb not just cytokines, but also a wide range of other molecules.[20] Thus, it is important to check the effects of perfusate adsorption in the clinical EVLP setting.

Donor lung reconditioning via perfusate adsorption during EVLP has led to a superior post-transplant lung function and decreased inflammatory response in this study. Donor driven alveolar macrophages have been thought to be the initiator of acute lung injury early after reperfusion. Cytokine release from alveolar macrophages further aggravates IR injury by the recruitment of recipient driven white blood cells into the allograft during the second phase of reperfusion.[31] Expectedly, macrophages are the predominant cell type in BWF after EVLP, whereas a neutrophilic response became evident after reperfusion in this study. We further analyzed the local inflammatory response using a multiplex cytokine assay. For most analyzed cytokines/chemokines, levels were lower in the treated lungs, with IL-1ra, IL-6, and IL-8 being markedly reduced. The inhibition of an inflammatory response during EVLP and after reperfusion is probably the mechanism underlying the protection against IR related injury. Interestingly, other than IL-1ra, the systemic inflammatory response to reperfusion did not differ significantly in this study. Similarly, the histological examination showed that the type of inflammatory response (recruitment of neutrophils into the lungs) did not differ between treated and untreated lungs post transplantation, while the extent of

recruitment (with evidence of emigration and accumulation in alveolar lumina) was overall higher in untreated control lungs. IL-1α, IL-1β, IL-1ra, and IL-18 are belonging to the IL-1 family cytokines that are being associated with acute and chronic inflammation.[32] Although the recombinant form of IL-1ra has been used as an anti-inflammatory drug in various inflammatory conditions, the soluble form is secreted by hepatocytes as an acute phase protein.[33] We believe that elevated IL-1ra plasma levels are a surrogate marker of enhanced inflammation regulated by proinflammatory cytokines. Apparently, the main mechanism that underlies our results with Cytosorb is an increased protection against injury related to adsorption of inflammatory mediators. Nonetheless, we could not rule out that the mechanism behind this observed injury protection may be different, improving lung compliance or redirecting the perfusate flow to better oxygenated area.

The main limitation of the present study is the lack of understanding the pathways involved in lung recovery during EVLP. Further studies are needed to understand the effects of the broad spectrum adsorber on the mechanisms underlying IR injury in lung transplantation. We have conducted the study with the least possible number of animals to reach a conclusion. The duration of reperfusion was only 4h, which is far shorter than the clinical definition of PGD. The non-selective nature of the adsorber should be carefully considered when planning treatments with anti-inflammatory molecules, such as IL-10. Taking into consideration of the limitations of the CytoSorb® adsorber, modified EVLP protocol warrants further studies in the clinical setting.

In summary, implementation of an additional cytokine adsorber has refined the standard EVLP protocol. Furthermore, cytokine removal during EVLP improved immediate post-transplant graft function and was associated with a less intense inflammatory response to

reperfusion in pigs. Further studies are warranted to understand the beneficial effects of perfusate adsorption during EVLP in the clinical setting.

Conflict of Interest Statement

The study was partially funded by the CytoSorbents Europe GmbH, including provision of the CytoSorb cartridges used for the experiments. The research fund was used to cover the costs related to animal experimentation and bench works. None of the authors have received remuneration related to the work they provided in this study. The authors were design the study and responsible for data collection, interpretation of data, writing the manuscript, and the decision to submit for publication. The funding source had no role in these processes. Additionally, Ilker Iskender received speaker fees from the funding source. Necati Citak was supported with a fellowship grant from the Scientific and Technological Research Council of Turkey. None of the authors has a financial relationship with a commercial entity that has an interest in the subject of the presented manuscript or other conflicts of interest to disclose.

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580
581 **FIGURE LEGENDS**
582 ***CENTRAL PICTURE:***
583 Perfusate adsorption during ex vivo lung perfusion improves post-transplant lung function
584 ***FIGURE 1: Pharmacokinetics of meropenem and methylprednisolone during the 12-***
585 ***hour of ex vivo lung perfusion period.***
586 Perfusate concentrations of meropenem (A) and methylprednisolone (B) were measured at
587 baseline, 1h, 3h, and in 3h intervals thereafter in samples collected during the initial trial.[15]
588 The perfusate concentrations of meropenem and methylprednisolone were markedly
589 decreased in the adsorber group compared with the control group (*** $p < 0.001$, between
590 groups). The drug removal effect of the adsorber was more prominent for
591 methylprednisolone than meropenem. Data are presented as percentage change from
592 baseline and expressed as mean ± standard deviation.
593 ***FIGURE 2: Ex vivo lung perfusion physiology and biochemistry.***
594 Pulmonary mechanics, vascular pressures, oxygenation function and perfusate gas analyses
595 were recorded hourly during the 6-hour EVLP period. EVLP physiology, determined by
596 peak airway pressure (A) and delta PO_2 (D) was significantly better in the treatment group.
597 EVLP biochemistry, determined by potassium (F), calcium (G), and lactate (I) levels was

598 also improved in the adsorber group. Pulmonary vascular resistance = Pulmonary artery
599 pressure – (Left atrial pressure / Flow). Delta PO_2 = left atrial pressure of oxygen –
600 pulmonary arterial pressure of oxygen. Data are expressed as mean ± standard deviation (*
601 $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, between groups).

602 ***FIGURE 3: Post-transplant physiological outcomes.***

603 Mean arterial pressure (A), dynamic compliance (B), and systemic arterial PaO_2/FiO_2 ratio
604 (C) were recorded at baseline, early after reperfusion and hourly thereafter. Dynamic
605 pulmonary compliance was significantly higher throughout reperfusion in the adsorber
606 group (B, * $p < 0.05$, between groups). Systemic arterial (C, $p = 0.1$) and pulmonary venous
607 (D, $p = 0.18$) oxygenation function were also better in the treatment group. After occlusion
608 of the contralateral anatomical structures, isolated allograft oxygenation function (E, $p =$
609 0.056) and dynamic compliance (F, * $p < 0.05$, between groups) continued to be better in
610 the treatment group. Matching results for each animal are presented. PaO_2/FiO_2, partial
611 pressure of arterial oxygen/fraction of inspired oxygen; PA, pulmonary artery; MB, main
612 bronchus; LTx, lung transplantation. Data are expressed as mean ± standard deviation.

613 ***FIGURE 4: Plasma levels of soluble Interleukin 1 receptor antagonist were significantly***
614 ***lower in the treatment group after reperfusion.*** Data are expressed as mean ± standard
615 deviation (** $p < 0.01$, between groups).

616 ***FIGURE 5: Correlation between tissue cytokine expression and histological assessment***
617 ***of the allografts.***

618 Matching results for each animal are presented for tissue cytokines, IL-1β and IL-8 (upper
619 panel) and microscopic lung injury, which was scored by two veterinary pathologists in a
620 blinded fashion (lower panel). Tissue expressions of IL-1β (A, $p = 0.056$) and IL-8 (B, $p =$

621 0.31) were lower in the treatment group. The overall microscopic lung injury scoring was
622 comparable between the two groups (C, p = 1). However, the presence of individual
623 neutrophils within alveolar lumina (D, p = 0.29) were lower, indicating a less intense
624 inflammatory response in the adsorber group. Data are expressed as mean mean ± standard
625 deviation. Comparison was made by Mann–Whitney U-test.

626 ***FIGURE 6:*** *Ex vivo* lung perfusion (EVLP) is characterised by the release of inflammatory
627 mediators into the perfusate. In the present study, we have tested the beneficial effects of
628 perfusate adsorption during EVLP on short-term post-transplant lung function in a pig
629 EVLP lung transplant model. This strategy resulted in preservation of post-transplant graft
630 function, by means of enhanced gas exchange and lung mechanics, via inhibition of
631 inflammatory response to ischemia-reperfusion injury in pigs.

632 ***Supplementary Figure:***

633 Histological features of lungs. A. Non-transplanted right lung after *ex vivo* lung perfusion
634 (EVLP) with adsorber. Histological changes are restricted to the apoptosis of leukocytes
635 within capillaries and in the peribronchiolar tissue (arrows). B-D. Transplanted left lung
636 from the control group at autopsy. Overall histological score of 9. B. Most vessels (arrows)
637 and capillaries (arrowheads) are packed with neutrophils. Score A3. C. Large vein with
638 evidence of neutrophil emigration, represented by extensive rolling of neutrophils along
639 endothelial cells (arrowheads) and the presence of neutropils within the perivascular tissue
640 (arrows). Score B3. D. Individual to groups of neutrophils are present within alveoli
641 (arrows). Score C3. Hematoxylin-eosin stain. Bars = 10 µm.

642

643

TABLE 1: The expression of cytokines and chemokines in perfusate at 3-hour and 6-hour of ex vivo lung perfusion.

Cytokine/Chemokine Levels in Perfusate				
Analytes	**Time points**	**Control (pg/mL)**	**Adsorber (pg/mL)**	**P-value**
IL-1α	**3h**	54 ± 49	6 ± 7	*** 0.029**
	6h	67 ± 50	1 ± 2	
IL-1β	**3h**	155 ± 60	51 ± 24	**** 0.006**
	6h	613 ± 540	81 ± 45	
IL-1ra	**3h**	294 ± 101	63 ± 32	***** 0.001**
	6h	1214 ± 535	35 ± 17	
IL-6	**3h**	719 ± 340	602 ± 352	**** 0.006**
	6h	2833 ± 821	791 ± 354	
IL-8	**3h**	17121 ± 8944	943 ± 766	**** 0.006**
	6h	28812 ± 11105	444 ± 343	
IL-10	**3h**	72 ± 30	24 ± 18	**** 0.006**
	6h	191 ± 107	4 ± 3	
IL-12	**3h**	185 ± 63	74 ± 46	**** 0.006**
	6h	404 ± 120	97 ± 51	
IL-18	**3h**	8068 ± 4416	245 ± 94	**** 0.002**
	6h	7980 ± 3647	131 ± 112	

IL, Interleukin; IL-1ra, IL-1 receptor antagonist. Data are expressed as mean ± standard deviation. Comparisons between the groups were made by two-way repeated measures analysis of variance.

TABLE 2: Inflammatory response in bronchial wash fluid samples at autopsy.

Cytokine/Chemokine Levels in Bronchial Wash Fluid			
Analytes	**Control (pg/mL)**	**Adsorber (pg/mL)**	**P-value**
IL-1α	16 ± 11	16 ± 16	**0.691**
IL-1β	358 ± 305	70 ± 157	**0.056**
IL-1ra	2411 ± 2200	218 ± 133	**** 0.008**
IL-6	898 ± 207	286 ± 255	** 0.016**
IL-8	1415 ± 990	301 ± 259	** 0.032**
IL-10	16 ± 12	6 ± 7	**0.151**
IL-12	125 ± 73	56 ± 81	**0.095**
IL-18	122 ± 124	9 ± 13	**0.067**

IL, Interleukin; IL-1ra, IL-1 receptor antagonist. Data are expressed as mean ± standard deviation. Comparisons between the groups were made by the Mann-Whitney test.

Supplementary Table: The quantitative presentation of the discordance between the two-pathologists related to the microscopical lung injury at the end of transplantation.

	Score A	Score B	Score C	Score Overall
Controls				
1	1	1 **[0.5-1]**	1	3
2	1.5 **[1-1.5]**	1	1	3.5
3	2	2	3	7
4	1	1	1.5 **[1.5-2]**	3.5
5	3	3	3	9
Adsorber				
1	2.5 **[2-2.5]**	1.5	0	4
2	1	2	1	4
3	2	2.5	2	6.5
4	1	1.5 **[1.5-2]**	2	4.5
5	1	1	0	2

Tissue samples were stained with hematoxylin and eosin and blindly assessed by two veterinary pathologists for any pathological changes. The scoring system to assess lung injury: A) Evidence of neutrophil recruitment into the lungs (1: moderate, 2: high, 3: vessels packed with neutrophils); B) Evidence of neutrophil emigration into the tissue (1: neutrophil rolling along vascular endothelial cells, 2: neutrophils within vessel walls, 3: neutrophils immediately outside vessels); and C) Evidence of neutrophils within the tissue, represented by the presence of individual neutrophils within alveolar lumina (1: in rare alveoli, 2: in occasional alveoli, 3: in numerous alveoli). The results showed a concordance in most of the cases with a very small disagreement in 5 tissues. Those cases showing disagreement were re-evaluated with particular emphasis on the inclusion of all areas of the sections, and a final definitive score was reached. The discordant results are given in red.